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Valproic Acid Upregulates NKG2D Ligand Expression through an ERK-dependent Mechanism and Potentially Enhances NK Cell–mediated Lysis of Myeloma[1]

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Abstract

Modulation of the antitumor immune response through the engagement of NKG2D receptors with their ligands (L) on targets represents a promising therapeutic approach against cancer. In this study, we tested the effect of valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, on the expression of NKG2D ligands in myeloma cells. We demonstrated that VPA was able to upregulate both protein and mRNA expression of major histocompatibility complex class I–related chain (MIC) A/B and UL16-binding protein (ULBP) 2 without any significant effect on the expression of ULBP1, ULBP3, and ULBP4 or induction of other natural killer (NK) cell ligands, such as NKp30-L, NKp44-L, and NKp46-L in myeloma cells. A ^{51}Cr release assay and degranulation assay indicated that the induction of MICA/B and ULBP2 augmented NK cell–mediated lysis of myeloma cells, which was abolished by the addition of a blocking NKG2D antibody. Activation of constitutively phosphorylated extracellular signal–regulated kinase (ERK) by VPA is essential for the up-regulation of MICA/B and ULBP2 expressions. Inhibition of ERK using ERK inhibitor PD98059 decreased both MICA/B and ULBP2 expressions and NK cell cytotoxicity. Furthermore, overexpression of constitutively active ERK in ARK resulted in increased MICA/B and ULBP2 expressions and enhanced NK cell lysis. These data indicate that increased sensitivity of VPA-treated myeloma cells to NK cell lysis is caused by higher NKG2D ligand expression, resulting from more active ERK signaling pathway. Our results provide evidence that targeting ERK signaling pathway may be an additional mechanism supporting the antimyeloma activity of HDAC inhibitors and suggest its possible immunotherapeutic value for myeloma treatment.

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Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by neoplastic accumulation of clonal secretory plasma cells in the bone marrow [1]. Recently, significant advances have been made by combining the immunomodulatory drugs as well as the proteasome inhibitors with autologous peripheral blood stem cell transplantation, which allows for long-term disease-free survival in the majority of transplant-eligible patients. However, MM still remains largely incurable with current therapeutic strategies [2]. The majority of patients with MM relapse with an average survival time of 4 to 7 years. These poor results led to the search for alternative treatment strategies such as adoptive natural killer (NK) cell transfer or other novel pharmaceuticals.

NK cells, as part of the innate immunity, substantially contribute to the elimination of virus-infected cells as well as antitumor immune

Abbreviations: APC, allophycocyanin; ATM, mutated ataxia-telangiectasia; CMA, concanamycin A; ERK, extracellular signal–regulated kinase; E/T, effector/target; FITC, fluorescein isothiocyanate; HDAC, histone deacetylase; L, ligand; MM, multiple myeloma; MIC, major histocompatibility complex class I–related chain; NK cell, natural killer cell; PE, phycoerythrin; ULBP, UL16-binding protein; VPA, valproic acid

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response [3]. NK cell activity is tightly regulated by a delicate balance between inhibitory and activating receptors that recognize ligands (L) on target cells [4]. Among the activating receptors involved in NK cell–mediated cytotoxicity, NKG2D is a C-type lectin-like activating receptor and expressed by NK cells, γ/δ T cells, and $CD8^+$ T cells in humans. NKG2D recognizes its ligands major histocompatibility complex class I–related chain (MIC) A, MICB, and UL16-binding proteins (ULBP) 1 to 4 in humans [5,6]. These NKG2D ligands are almost not expressed in normal cells. However, they are constitutively expressed in many malignant cells, such as those in colon, liver, ovary, and hematological malignancies [7–11], and are upregulated on cellular stress, cell transformation, and viral infection [12,13]. The efficiency of NKG2D-mediated cytotoxicity by NK cells has been shown to correlate with the expression levels of NKG2D ligands on target cells [10]. Thus, the modulation of the interaction between NKG2D and its ligands is thought to play an important role in tumor rejection and surveillance.

Characterized by their anticancer properties, histone deacetylase (HDAC) inhibitors have recently attracted much attention. HDAC inhibitors induce an increased acetylation of DNA-associated histone proteins, leading to cell cycle arrest, redifferentiation, and apoptosis in a broad range of malignant cells [8]. With a favorable pharmacologic profile and well-tolerated side effects, the well-known antiepileptic drug valproic acid (VPA) has been demonstrated to mediate HDAC inhibition and to display antineoplastic activity safely *in vivo* [14]. In addition, VPA has been found to sensitize tumor cells to NK cell–mediated lysis dependent on NKG2D signaling [15,16]. Increased NK cell–mediated lysis after VPA treatment was initially found in neuroblastoma cells [17]. This result was later confirmed in hepatoma cells and leukemic cells [8,16]. These data suggest that VPA might be a valuable new and tumor-selective drug by enhancing immune surveillance for treatment of MM.

In this study, we investigated the potential role of NKG2D in immune surveillance against MM by analyzing expressions of the various NKG2D ligands on panels of myeloma cell lines and patient myeloma cells after treatment with VPA, thus evaluating its impact on recognition of tumor cells by cytotoxic effectors. We show that treatment of human myeloma cells with VPA upregulates expression of NKG2D ligand, leading to a significantly increased NK cell–mediated lysis of tumor cells due to enhanced NKG2D engagement. Constitutively active phosphorylated extracellular signal–regulated kinase (pERK) but not pAKT by VPA is essential for the up-regulation of NKG2D ligand in myeloma cells. Therefore, VPA in combination with adoptive NK cell transfer as a new treatment option might be especially useful for control of residual minimal disease by increasing susceptibility of myeloma cells to immune surveillance.

Materials and Methods

Cell Culture

ARK, OPM2, and OCI-MY5 myeloma cell lines were obtained from American Type Cell Collection (ATCC, Manassas, VA) and were cultured in RPMI 1640 medium (Invitrogen, Frederick, MD) supplemented with 10% FBS (Invitrogen) and 1% penicillin streptomycin–glutamine in an atmosphere of 5% carbon dioxide at 37°C. Peripheral blood samples and bone marrow samples were obtained from patients and healthy donors. The institutional review board of Tongji University School of Medicine approved these research studies and all subjects provided written consent approving use of their samples for research purposes. $CD56^+/CD3^-$ NK cells and $CD138^+$ myeloma cells were isolated from peripheral blood and bone marrow using magnetic bead selection (Miltenyi Biotech, Auburn, CA). All selected cells were more than 94% pure.

Reagents and Antibodies

Histone deacetylase inhibitor VPA was purchased from Sigma-Aldrich (Shanghai, China). Cell surface protein expression was determined by standard flow cytometry using unconjugated or fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, and allophycocyanin (APC)–conjugated antibodies (Abs) to CD3, CD56, CD138, MICA/B (BD Pharmingen, San Diego, CA), ULBP1 to ULBP4, CD48, CS1, CD155, CD112, CD28, AICL(CLEC2B), LFA-3 (CD58; R&D Systems, Minneapolis, MN). Annexin V and 7AAD were from BD Pharmingen. The recombinant (r) NKp30/human IgG1 Fc chimera, rNKp44/human IgG1 Fc chimera, and rNKp46/human IgG1 Fc chimera were purchased from R&D Systems. Human IgG was purchased from Caltag Laboratories (Burlingame, CA). The procedure for staining cells with Ig fusion proteins has been described [18]. The blocking anti-NKG2D monoclonal antibody (mAb) and mouse IgG1 were purchased from R&D Systems. The following primary Abs were used for Western blot analysis: p44/42 mitogen-activated protein kinases (MAPK) (ERK1/2), anti–phospho-ERK1/2, AKT, anti–phospho-AKT (Ser473; Cell Signaling, Beverly, MA), MICA/B, ULBP1 to ULBP4 (R&D Systems), and polyclonal β-actin (Sigma-Aldrich). The ERK inhibitor PD98059 was from Calbiochem (Darmstadt, Germany).

Flow Cytometry

Cell surface expression of protein was tested using flow cytometry. Cells were washed twice with cold phosphate-buffered saline (PBS) and stained with indicated unconjugated or FITC-, PE-, APC-conjugated Abs or isotype Ab for 30 minutes at 4°C. After incubation for 30 minutes at 4°C, cells were washed three times with PBS and suspended in PBS. Samples were examined on a flow cytometer (FACSCalibur; Becton Dickinson Medical Devices, Shanghai, China), and the data were analyzed using FCS Express 3 Flow Cytometry software (De Novo Software, Los Angeles, CA). In all experiments, cells were analyzed for NKG2D ligand expression or other NK cell ligand expression by gating on annexin V and 7AAD double negative cells.

Western Blot Analysis

Cells were collected and resuspended in lysis buffer [30 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM phenylethylsufonyl fluoride, 5 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, and 10 mg/ml aprotinin] for 15 minutes at 4°C. The suspension was then vortexed and centrifuged at 10,000*g* for 10 minutes. The supernatant was collected and mixed with an equivalent volume of 2× protein loading buffer containing 2-mercaptoethanol and boiled for 5 minutes before loading onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Upon completion of electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary Abs for 1 hour at room temperature. Membranes were washed with PBS (pHzz 7.4) containing 0.05% Tween-20 and incubated with HRP-conjugated secondary Ab for 1 hour. Protein bands were visualized by enhancing chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). β-Actin was used to normalize the amount of protein in each sample.

RNA Extraction and Real-time Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The quality and concentration of the extracted RNA were determined by measuring light absorbance at 260 nm (A_{260}) and calculating the A_{260}/A_{280} ratio. A measure of 2-μg sample of total RNA was then reverse transcribed into cDNA. One microliter of cDNA was then subjected to polymerase chain reaction (PCR) using specific primers. To internally standardize the levels of gene expression, we used the β-actin housekeeping gene. Amplifications were performed with the ABI PRISM 7000 Sequence Detection System in a 50-μl final volume using 40 cycles of a two-step PCR (15 seconds at 94°C and 60 seconds at 60°C) after initial denaturation (95°C for 15 minutes). The primer sequences are given as follows: MICA sense, 5′-CAGGGACTTGACAGGGAAC-3′ and antisense, 5′-CCTCTCCTCGGCAAATCCT-3′; MICB sense, 5′-ACCGAGGACTTGACAGAGA-3′ and antisense, 5′-CCGCTGATGTTTTCTTCT-3′; ULBP1 sense, 5′-TCTGTGCCTCCCGCTTCTG-3′ and antisense, 5′-GCCTTGGGTTGGGTTGTGC-3′; ULBP2 sense, 5′-GCAACAAGACAGTCACACC-3′ and antisense, 5′-AGCAGGGGAGGATGATGAG-3′; ULBP3 sense, 5′-TCCATCGGCTTCACACTCA-3′ and antisense, 5′-GTGAGGTCCAGAGCCAGGT-3′; ULBP4 sense, 5′-CCTCCTGGGGAAGAAGGTAT-3′ and antisense, 5′-GGATTTCACTCCATTGGTGGC-3′.

Degranulation Assay

NK cell–mediated cytotoxicity was evaluated using the lysosomal marker CD107a as previously described [19]. In brief, NK cells were isolated from healthy donors. Drug-treated patient myeloma cells were incubated with NK cells at an effector/target (E/T) ratio of 2.5:1 in a U-bottom 96-well tissue culture plate with complete medium at 37°C and 5% CO_2 for 2 hours. Thereafter, cells were washed with PBS and

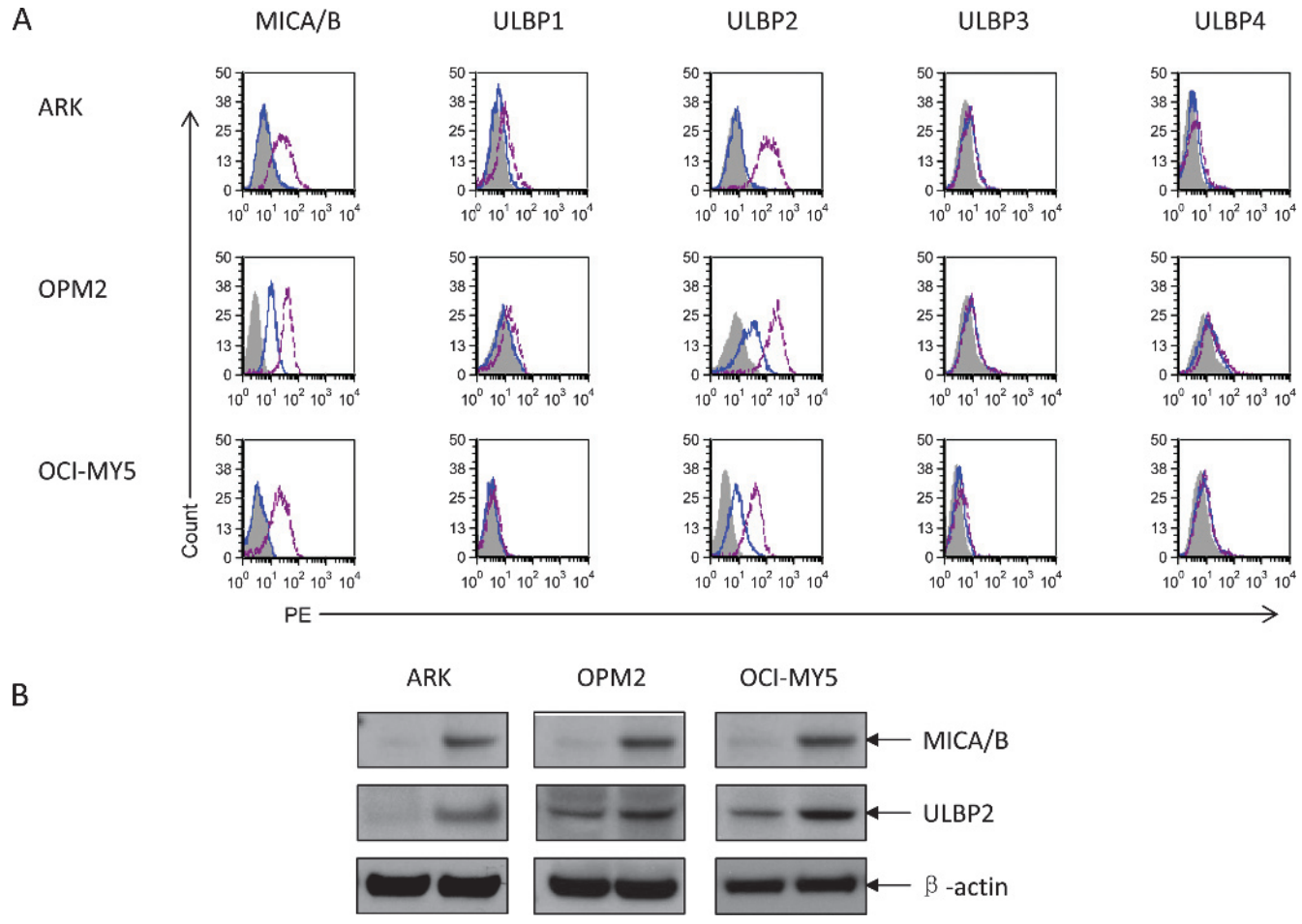

Figure 1. Expression of NKG2D ligands and other NK cell ligands on human myeloma cell lines and patient myeloma cells after treatment with 1 mM VPA for 48 hours. (A) Selective up-regulation of MICA/B and ULBP2, without any significant effect on ULBP1, ULBP3, and ULBP4 levels after VPA treatment on annexin V and 7AAD negative non-apoptotic ARK, OPM2, and OCI-MY5 cells. Histograms of NKG2D ligand expression on three myeloma cell lines are shown. Gray lines represent untreated cells; blue lines represent isotype control; purple lines represent VPA-treated cells. (B) Up-regulation of protein expression of MICA/B and ULBP2 on VPA-treated ARK, OPM2, and OCI-MY5 cells by Western blot analysis. (C) No significant changes of expressions of other NK cell ligands, such as NKp30-L, NKp44-L, NKp46-L, CD48, CS1, CD155, CD112, CD28, AICL(CLEC2B), and LFA-3(CD58), were seen on ARK cells after exposure to VPA. Gray lines represent untreated cells; blue lines represent isotype control; purple lines represent VPA-treated cells. (D) Selective up-regulation of MICA/B and ULBP2 by VPA treatment on annexin V and 7AAD negative non-apoptotic patient myeloma cells ($n = 4$). Histograms of NKG2D ligand expression on patient myeloma cells are shown. Gray lines represent untreated cells; blue lines represent isotype control; purple lines represent VPA-treated cells.

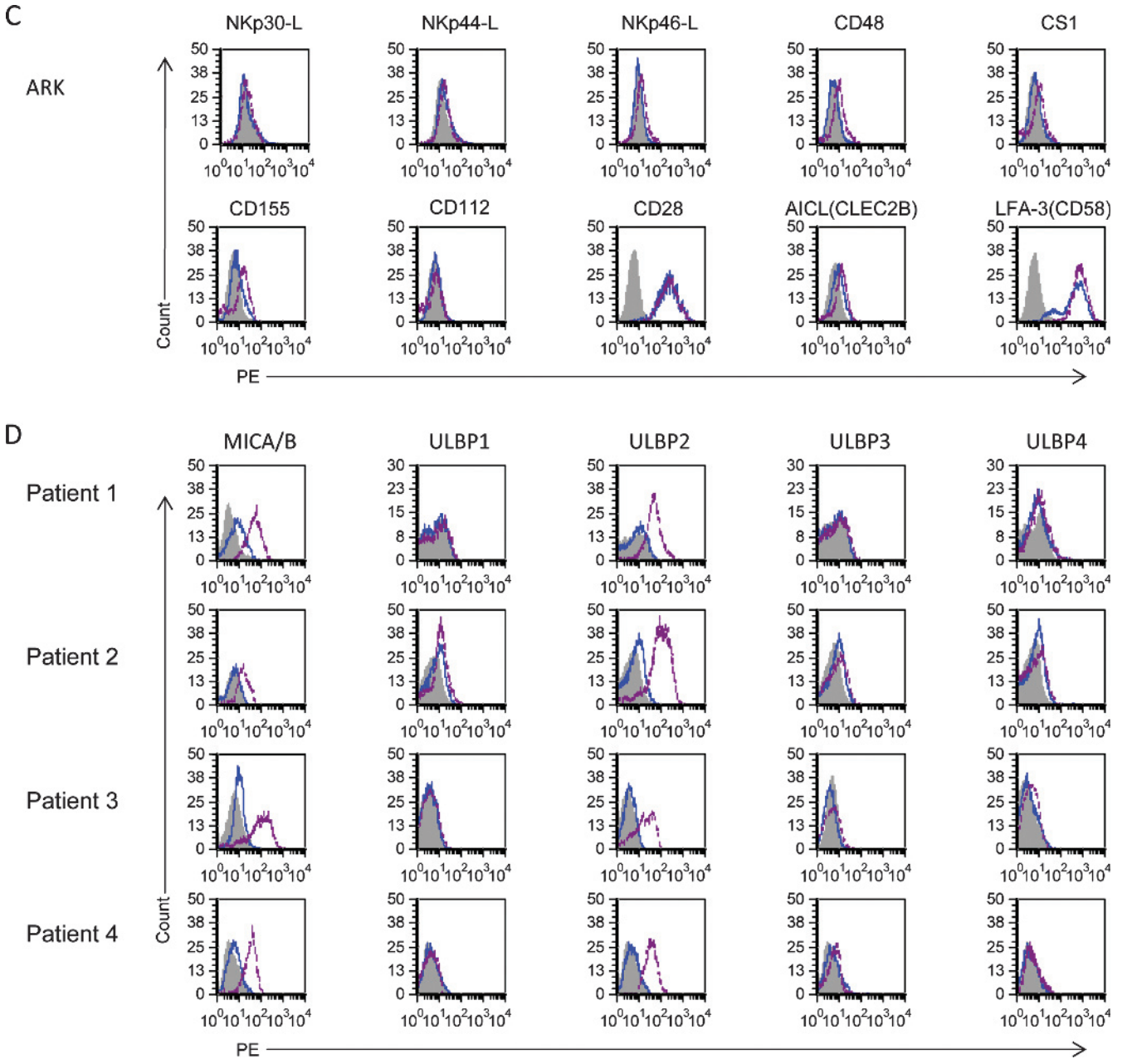

Figure 1. (*continued*).

incubated with APC-conjugated anti-CD107a (or APC-conjugated IgG) for 45 minutes at 4°C. Cells were then stained with anti-CD3 PE or anti-CD56 FITC to gate the $CD3^{-}CD56^{+}$ NK cell population. In some experiments, cells were pretreated with anti–NKG2D-neutralizing mAbs or isotype control for 30 minutes at room temperature. Fluorescence was analyzed using a flow cytometer, and the data were analyzed using FCS Express 3 Flow Cytometry software.

Cytolytic Assay

NK cell cytotoxicity against myeloma cells was analyzed using a standard ^{51}Cr release assay at the indicated E/T ratio. Target cells were incubated with VPA or control medium for 48 hours. NK cells isolated from healthy donors were added to the target cells and incubated for 4 hours at 37°C. In blocking experiments, the blocking anti-NKG2D mAb and mouse IgG1 as isotype control were added to the NK cells at 10 μg/ml 30 minutes before the coculture. To block perforin/granzyme-mediated killing, we pretreated NK cells for 2 hours in 100 nM concanamycin A (CMA; Sigma-Aldrich). To test the role of Fas-L and TRAIL-mediated cytotoxicity, we added anti–Fas-L (5 μg/ml), anti-TRAIL (2.5 μg/ml), or control IgG1 Ab (5 μg/ml) to effectors at the time of plating and left it in the wells for the rest of the assay. The NK cell–sensitive cell line K562 was used as positive control. Specific lysis percentage was calculated as (test release − spontaneous release)/(maximal release − spontaneous release) × 100. All experiments were performed in triplicate wells.

Transfection and Plasmid

The constitutively active ERK expression plasmid was purchased from Biovector Science Lab (Beijing, China). Plasmids included pcDNA3.1 (empty vector) as the control and constitutively active ERK vector (pcDNA3.1 ERK). A total of 2×10^6 ARK cells were

transfected with 2 μg of plasmid from Amaxa Nucleofection Technology (Cologne, Germany). Transfection efficiency was approximately 72% as determined using green fluorescent protein, and maximal levels of protein expression were observed between 72 and 96 hours.

Statistical Analysis

Results of experimental points obtained from multiple experiments were reported as means ± SD. Statistical analysis was performed using the SPSS 15.0 software. Difference between untreated and VPA-treated groups was performed using a paired Student's *t* test. Differences among the groups were performed using one-way analysis of variance followed by the Turkey post hoc multiple comparison tests. A value of $P < .05$ was considered statistically significant.

Results

Histone Deacetylase Inhibitor VPA Induces NKG2D Ligand Protein Expression in Patient Myeloma Cells

To investigate the effect of VPA on the surface expression of NKG2D ligands in myeloma cells, we initially performed a flow cytometric analysis by gating on annexin V and 7AAD negative non-apoptotic ARK, OPM2, and OCI-MY5 MM cells before and after treatment with VPA. As shown in Figure 1*A*, treatment of ARK, OPM2, and OCI-MY5 cells with this inhibitor was able to upregulate expression of MICA/B and ULBP2 without any significant effect on ULBP1, ULBP3, and ULBP4 levels. Up-regulation of protein expression of MICA/B and ULBP2 was further confirmed by Western blot analysis (Figure 1*B*). VPA treatment did not significantly affect surface expression of other NK cell ligands, such as NKp30-L, NKp44-L, NKp46-L, CD48, CS1, CD155, CD112, CD28, AICL(CLEC2B), and LFA-3(CD58) of ARK cells, as assessed by flow cytometric analysis (Figure 1*C*). Similar results were obtained by stimulating OPM2 and OCI-MY5 cells with VPA (data not shown). We next examined the effect of VPA on the surface expression of NKG2D ligands in patient MM cells. Increased expressions of MICA/B and ULBP2 were observed in VPA-treated patient MM cells (Figure 1*D*, $n = 4$). Elevation of ULBP1, ULBP3, and ULBP4 expression levels following VPA treatment was not observed in primary MM cells from four MM patients, which is consistent with the result from MM cell lines. VPA also did not have a significant effect on expression of other NK cell ligands in patient MM cells (data not shown). These data showed that HDAC inhibitor VPA is able to enhance the protein expression of

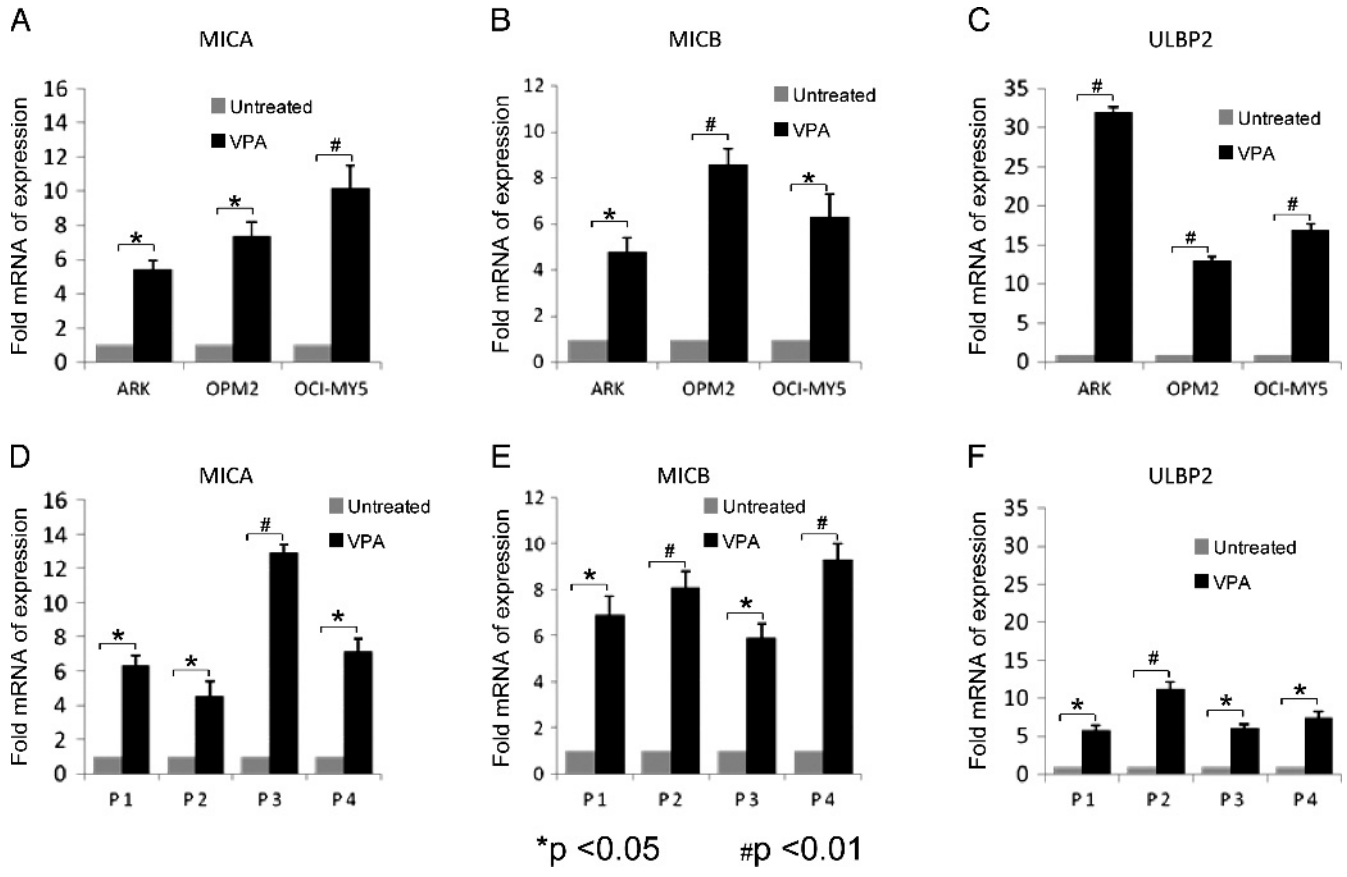

Figure 2. (A) Effect of VPA on mRNA expression of MICA on myeloma cell lines. (B) Effect of VPA on mRNA expression of MICB on myeloma cell lines. (C) Effect of VPA on mRNA expression of ULBP2 on myeloma cell lines. (D) Effect of VPA on mRNA expression of MICA on patient myeloma cells. (E) Effect of VPA on mRNA expression of MICB on patient myeloma cells. (F) Effect of VPA on mRNA expression of ULBP2 on patient myeloma cells. Effect of VPA on mRNA expression of NKG2D ligands on human myeloma cell lines and patient myeloma cells. ARK, OPM2, and OCI-MY5 cells and four patient myeloma cells were treated with 1 mM VPA for 48 hours. The gene expression levels relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined using real-time PCR. Each column and error bar represents the mean ± SD of the ratios of the NKG2D ligand mRNA level to the GAPDH mRNA level. The result was from three independent experiments. $^{*}P < .05$; $^{\#}P < .01$. P represents patient.

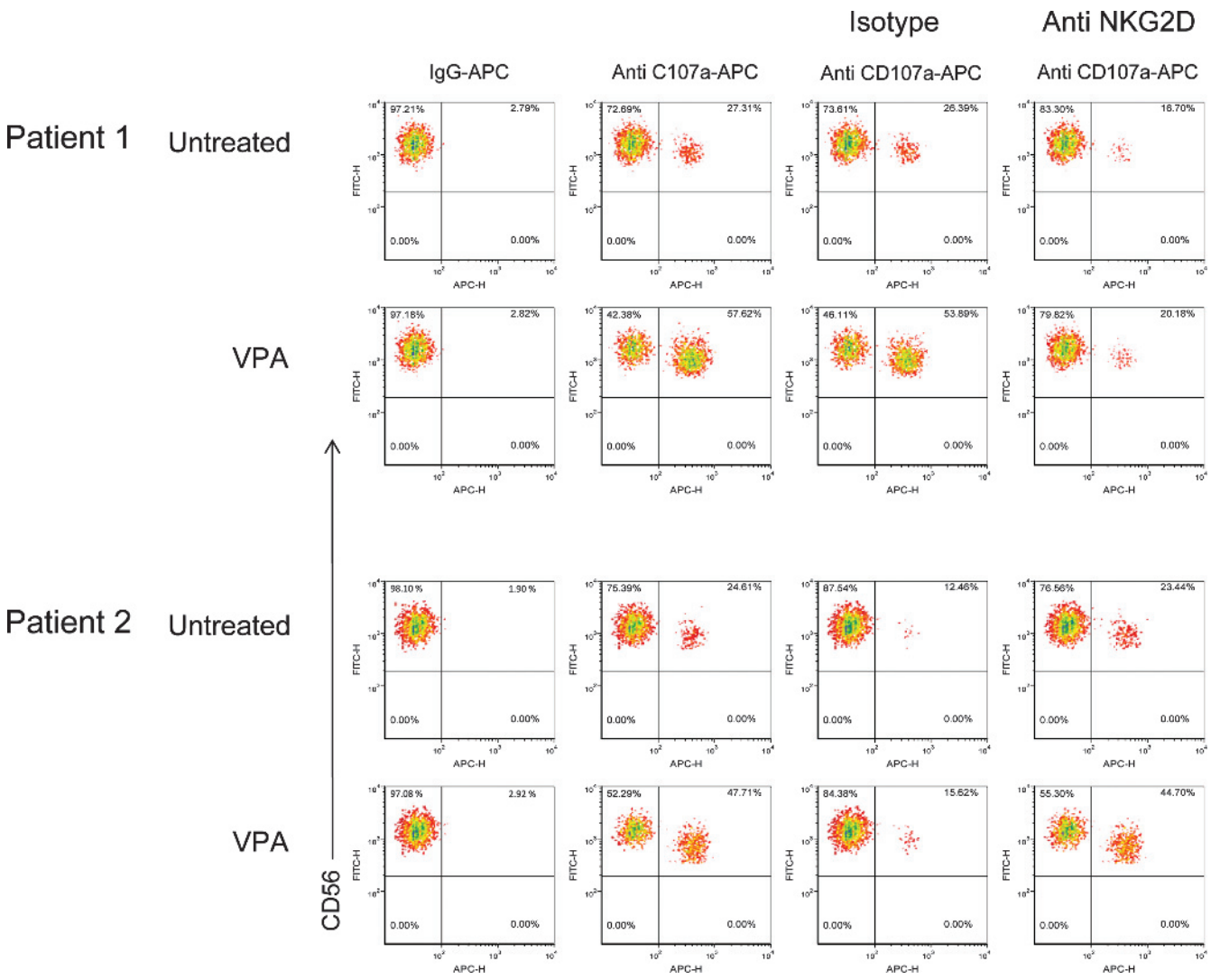

Figure 3. Effect of VPA on NK cell degranulation against patient myeloma cells. NK cells prepared from peripheral blood mononuclear cells (BMCs) of healthy donors were incubated with patient myeloma cells, untreated or treated with 1 mM VPA for 48 hours, used as targets in a degranulation assay. The assay was performed at an E/T ratio of 2.5:1. After a 2-hour incubation at 37°C, cells were stained with anti-CD56, anti-CD3, and anti-CD107a mAbs. Cell surface expression of CD107a was analyzed on $CD56^{+}CD3^{-}$ NK cells by flow cytometry. To evaluate the role of NKG2D, we performed the assay on NK cells that are in parallel treatment with blocking anti-NKG2D Ab or isotype. Results represent two of the four patient myeloma cells.

NKG2D ligands, MICA/B and ULBP2, in both myeloma cell lines and patient myeloma cells.

VPA Induces NKG2D Ligand mRNA Expression in Myeloma Cells

We next examined whether up-regulation of MICA/B and ULBP2 protein expressions by VPA treatment could be the consequence of up-regulation of their mRNA expression in MM cells. We treated ARK, OPM2, and OCI-MY5 cells with VPA, and mRNA expression of NKG2D ligand was analyzed by real-time PCR. Upon VPA treatment for 48 hours at 1 mM, a significant increase in MICA and MICB expression ranging from 5- to 11-fold when compared with the control was observed in the three tested MM cell lines (Figure 2, *A* and *B*). ULBP2 mRNA expression also significantly increased in the three cell lines (Figure 2*C*). It was important to note that no significant changes of ULBP1, ULBP3, and ULBP4 transcripts were examined in the three tested cell lines after VPA treatment (data not shown). Furthermore, we investigated whether treatment with VPA had an effect on mRNA expressions of MICA, MICB, and ULBP2 in patient MM cells. Similar results were obtained by stimulating four patient MM cells with VPA. Various degrees of up-regulation of MICA, MICB, and ULBP2 mRNA expressions were observed in patient myeloma cells following VPA treatment for 48 hours (Figure 2, *D–F*). There was no significant change in ULBP1, ULBP3, and ULBP4 transcripts in patient MM cells after exposure to VPA (data not shown). These results strongly suggest that VPA is able to enhance MICA, MICB, and ULBP2 mRNA expressions in human myeloma cells.

VPA Treatment Enhances NK Cell Degranulation against Patient Myeloma Cells

To investigate whether the up-regulation of NKG2D ligand on patient myeloma cells could lead to an activation of cytolytic NK cells, we analyzed the degranulation of NK cells against MM cells by evaluating the expression of the lysosomal marker CD107a on

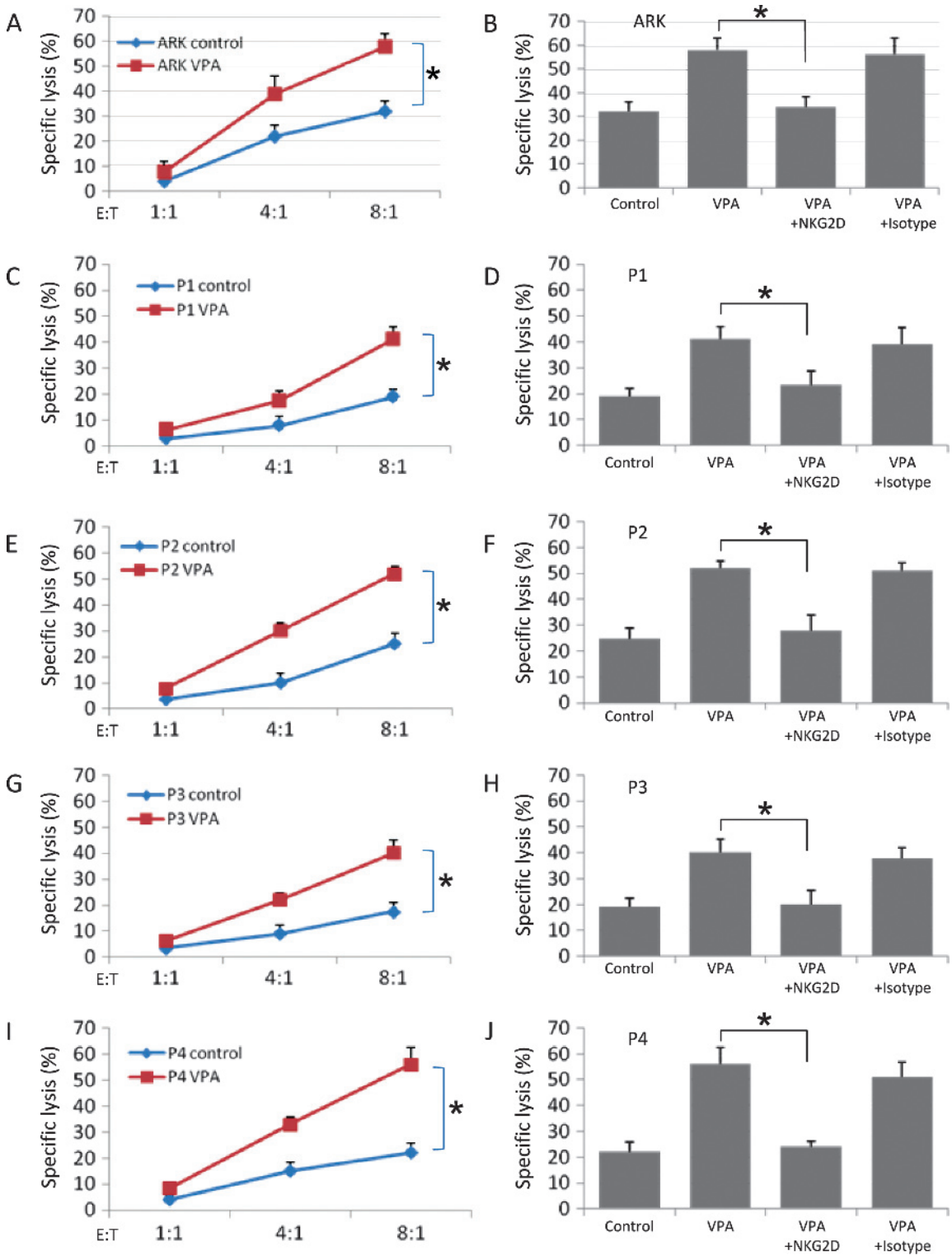

Figure 4. Enhanced NK cell–mediated lysis of human myeloma cell lines and patient myeloma cells following treatment with VPA. (A) ARK cells were incubated with or without 1 mM VPA for 48 hours, then examined for their sensitivity to killing by NK cells from a healthy donor. (B) NK cells were incubated in the presence of anti-NKG2D Abs or medium alone for 30 minutes, then used for chromium release assay against VPA-treated ARK cells at E/T 8:1. (C, E, G, I) Primary myeloma cells from four patients were incubated with or without 1 mM VPA for 48 hours, and the cytotoxicity of NK cells from healthy donors against patient myeloma cells was assessed by chromium release assay. (D, F, H, J) NK cells were incubated in the presence of anti-NKG2D Abs or medium alone for 30 minutes, then used for chromium release assay against VPA-treated patient myeloma cells at E/T 8:1. (K) The cytotoxicity assays (E/T = 8:1) were performed in the presence and absence of the perforin inhibitor CMA (100 nM), anti–Fas-L (5 μg/ml), anti-TRAIL mAb (2.5 μg/ml), or isotype Ab (5 μg/ml) against VPA-treated ARK cells. Data are presented as means (±SD) of triplicate samples. P represents patient. *$P < .05$; **$P > .05$.

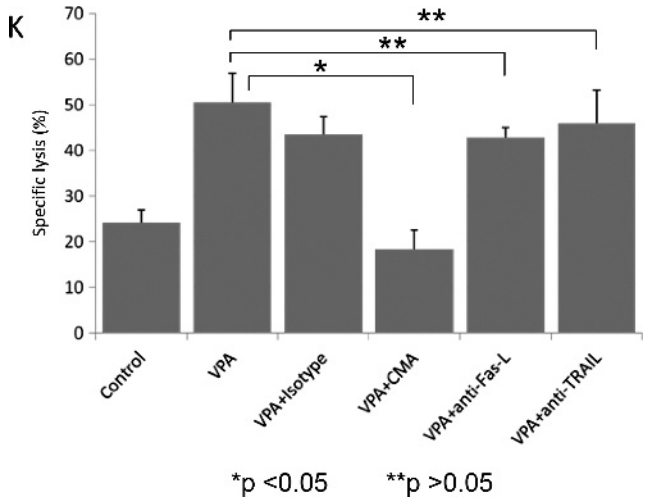

Figure 4. (*continued*).

the effector cells by flow cytometry analysis (FACS) [19]. Purified NK cells isolated from health donors were co-incubated with patient myeloma cells treated with or without VPA. NK cells were triple stained with APC-conjugated anti-CD107a mAb and with FITC-conjugated anti-CD56 mAb together with PE-conjugated anti-CD3 mAb. A representative experiment is depicted in Figure 3. A small percentage of NK cells expressed CD107a at the cell surface when co-incubated for 2 hours with untreated controls. Of note, the percentage of $CD107a^+$ NK cells was significantly increased upon interaction with VPA-treated patient myeloma cells when compared with untreated controls (mean ± SD: 52.0 ± 4.2% *vs* 23.9 ± 2.6%, $n = 4$, $P < .01$). The assay was performed at an E/T ratio of 2.5:1, and similar results were obtained using different E/T ratios (data not shown). Furthermore, this increase of degranulation was considerably reduced in the presence of a blocking anti-NKG2D mAb, whereas no significant change of degranulation was observed upon treatment with isotype control, suggesting that this enhancement of degranulation was dependent on NKG2D activation (Figure 3). NKG2D-blocking mAb partially affected basal degranulation, indicating that constitutive NK cell degranulation also involves this activating receptor. Our data suggest that augmented expressions of MICA/B and ULBP2 in patient myeloma cells treated with VPA enhance NK cell degranulation by promoting NKG2D recognition.

VPA Treatment Augments NK Cell Lysis of Myeloma Cells through NKG2D/NKG2D Ligand Interaction

Because engagement of NKG2D receptors by MICA/B and ULBP ligands triggers antitumor cytotoxicity of NK cells, we studied the functional relevance of enhanced MICA/B and ULBP expressions on ARK cells after VPA treatment. NK cells isolated from healthy individuals were examined against ARK cells treated with or without VPA using ^{51}Cr release assays. NK cell lysis of the K562 cell line was used as the positive control, and it resulted in approximately 85% lysis (E/T 8:1; data not shown). Treatment of ARK cells with VPA showed higher sensitivity to NK cell lysis than untreated control cells (mean ± SD: 58 ± 5% *vs* 32 ± 4% at E/T 8:1, $P < .05$; Figure 4*A*). The enhancing effect of VPA was blocked by NK cells pretreated with anti-NKG2D mAb (Figure 4*B*). VPA treatment of myeloma cells obtained from four MM patients also significantly increased the cytotoxicity of NK cells than that of the untreated controls. Using the same number of NK cells, the mean lysis of VPA-treated patient myeloma cells by NK cells was 47 ± 8% at an E/T ratio of 8:1, compared to untreated MM cells (mean ± SD: 21 ± 3%, $n = 4$, $P < .01$; Figure 4, *C*, *E*, *G*, and *I*). Importantly, an increase in the killing of VPA-treated patient MM cells was also strongly reduced with the addition of anti-NKG2D mAb to the cytolytic assay (Figure 4, *D*, *F*, *H*, and *J*). To identify the cytotoxic pathway used by NK cells, we examined the involvement of the perforin/granzyme and death receptor ligand pathways. The primary mechanism of NK cell killing of myeloma cells was perforin/granzyme-mediated, as the perforin/granzyme inhibitor CMA greatly inhibited killing, whereas blockings of TRAIL and Fas-L did not have a significant impact on NK cell lysis of myeloma (Figure 4*K*). A ^{51}Cr release assay indicated that the treatment of NK cells with VPA did not affect their cytotoxicity against ARK cells (data not shown), thus suggesting that NK cells can exert cytotoxicity against myeloma cells in the presence of this drug. These findings support NK cell activation through NKG2D/NKG2D ligand interaction as the possible mechanism involved in the increased lysis of myeloma cells after *in vitro* treatment with VPA. Our data also indicated NK cells' predominant role in the perforin/granzyme-mediated killing of myeloma cells.

Possible Mechanism for Increased NKG2D Ligand Expression in VPA-treated Myeloma Cells

Both ERK and AKT were shown to be involved in the regulation of expression of NKG2D ligands [20,21]. For these reasons, both ERK and AKT signaling pathways were studied for their constitutive activation status in untreated ARK cells and VPA-treated ARK cells. Western blot analysis showed a stronger constitutive pERK but not AKT in drug-treated ARK cells than those in the untreated cells (Figure 5*A*). To confirm the role of ERK signaling pathway in NKG2D ligand expression, we performed flow cytometric analysis and cytotoxicity assay on ERK inhibition of ARK cells and VPA-treated ARK cells using 20 μM PD98059. Treatment of both untreated ARK cells and VPA-treated ARK cells with PD98059 showed only minimal effect on cell viability (data not shown). It, however, suppressed their ERK activation status, reduced MICA/B and ULBP2 surface expression, and impaired NK cell lysis (52.3 ± 3.2% *vs* 26.7 ± 2.8% for VPA-treated cells *vs* VPA plus PD98059-treated cells, respectively, $P < .05$; Figure 5, *B*–*E*). Exposure of VPA-treated ARK cells to PD98059 had no effect on ULBP1, ULBP3, and ULBP4 ligand expressions (data not shown). These results were further confirmed by transfecting ARK cells with constitutively active ERK. Transfection of ARK cells with constitutively active ERK resulted to significantly higher MICA/B and ULBP2 expressions when compared with parental ARK cells (Figure 6, *A* and *B*). It also enhanced NK cell lysis by 2.1-fold, compared with parental ARK cells (Figure 6*C*). Furthermore, blocking NKG2D with anti-NKG2D mAb inhibited NK cell lysis (Figure 6*D*). These findings suggest that increased sensitivity of VPA-treated ARK cells to NK cell lysis is caused by higher NKG2D ligand expression, resulting from more active ERK but not AKT signaling pathway.

Discussion

Activation of the immune system, mediated by engagement of NKG2D receptor with its ligands, plays an important role in immunosurveillance of cancer [22,23]. Tumor NKG2D ligand expression is

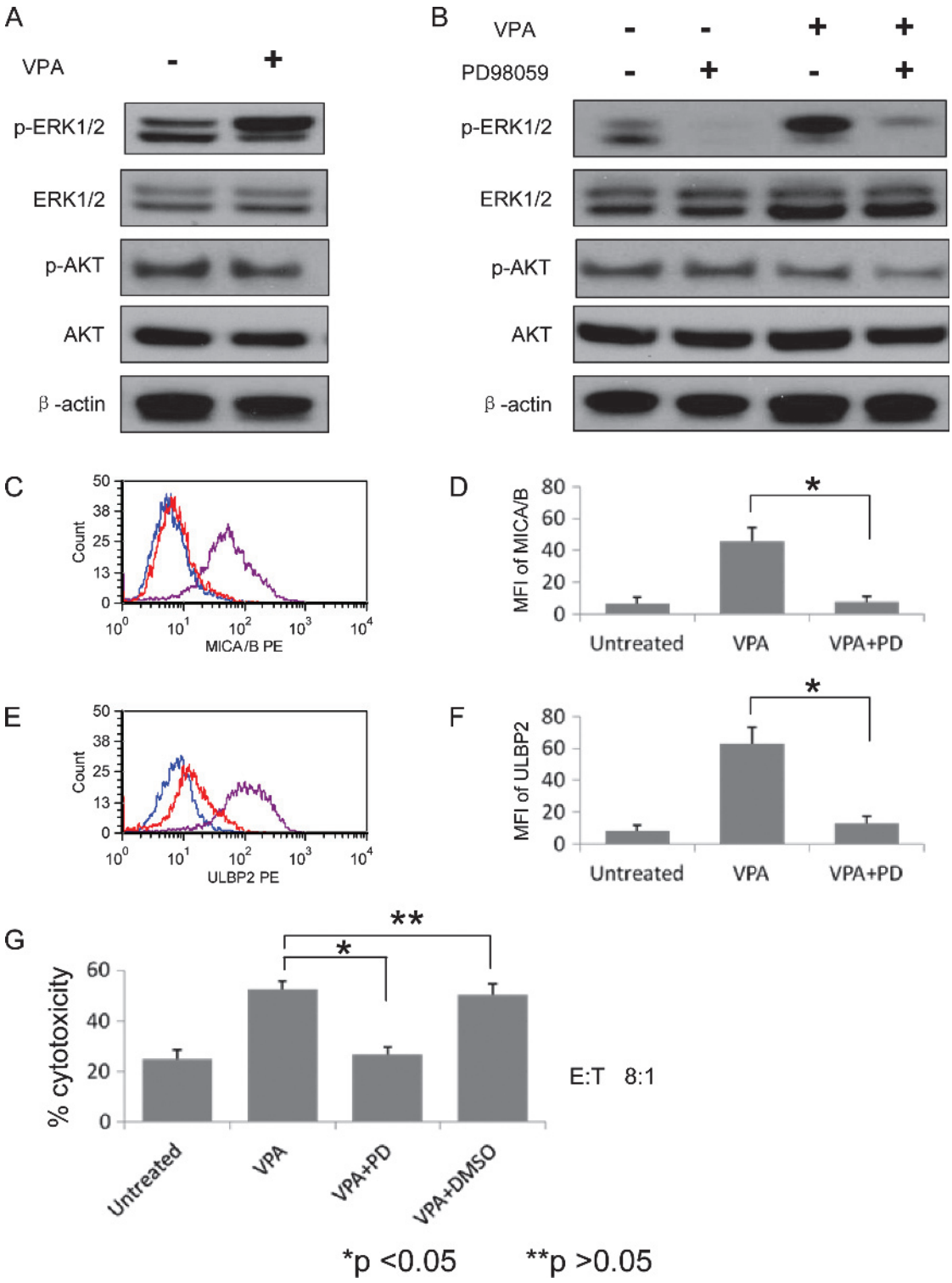

Figure 5. Mechanism of increased NKG2D ligand expression in VPA-treated ARK cells. (A) Constitutive activation of pERK in VPA-treated ARK cells compared with that of the untreated cells is shown. The same amount of cell extracts prepared from the VPA-treated or medium-alone cell cultures was used for Western blot analyses. (B) Western blot analysis of VPA-treated ARK cells after specific inhibition of ERK activation using PD98059. Data are representative of three experiments. (C–F) MICA/B and ULBP2 expressions in VPA-treated ARK cells after a 48-hour treatment with PD98059 (PD) using flow cytometric analysis. Histograms and mean fluorescence intensity (MFI) levels of MICA/B and ULBP2 expressions on ARK cells are shown. Blue lines represent untreated cells; purple lines represent VPA-treated cells; red lines represent VPA plus PD98059–treated cells. Results are representative of three different experiments. Error bars indicate ±SD. (G) NK cell cytotoxicity of ARK cells treated with or without VPA after a 48-hour treatment with PD98059 or DMSO alone. Results are representative of three different experiments. Error bars indicate ±SD. *$P < .05$; **$P > .05$.

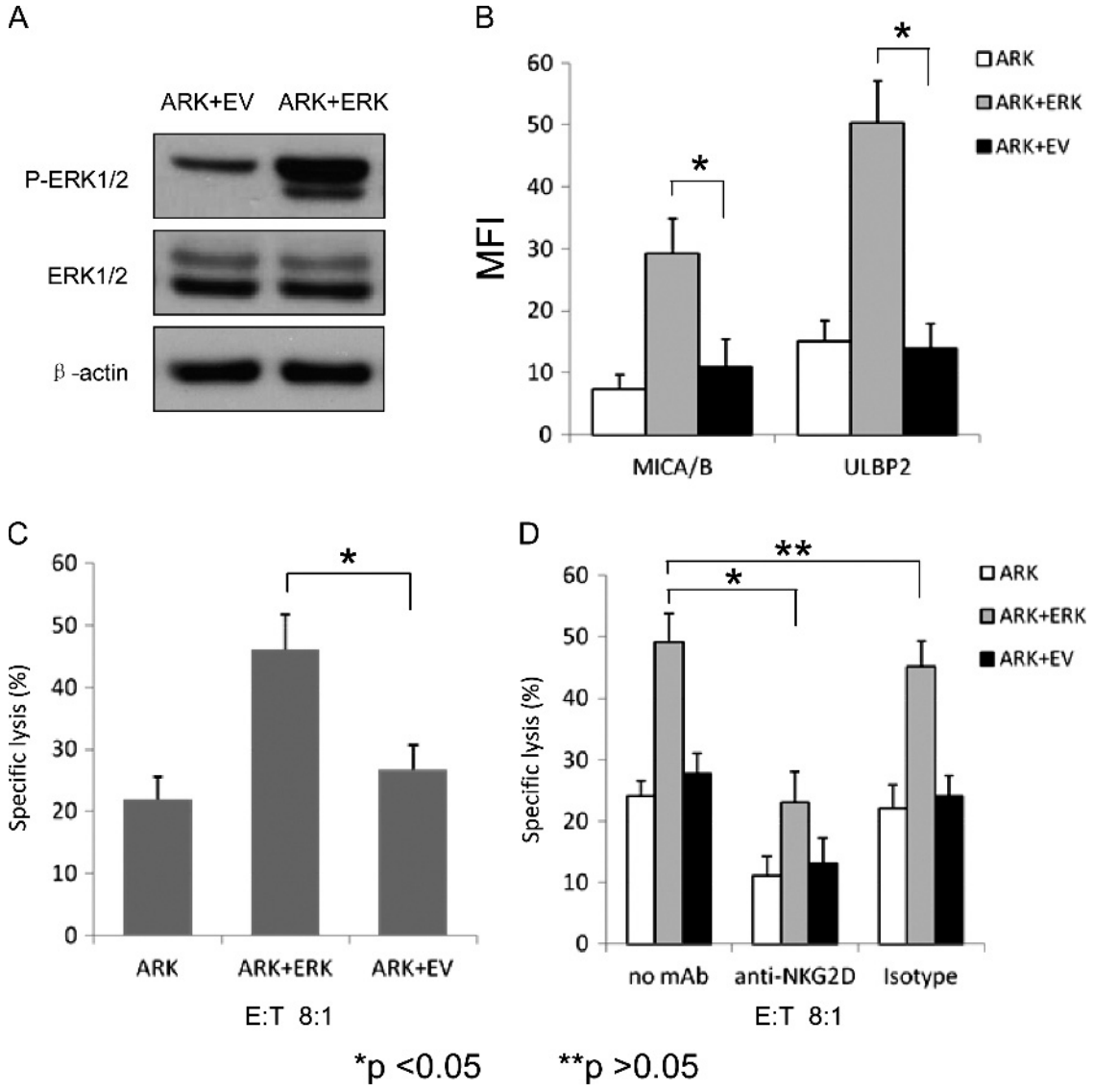

Figure 6. ERK activation is responsible for increased NKG2D ligand expression. (A) ERK activation was assessed by Western blot analysis of constitutively active ERK-transfected ARK (ARK + ERK). Empty vector (EV)–transfected ARK cells (ARK + EV) were used as control. (B) Flow cytometric analysis on NKG2D ligand expression after transfection of ARK cells with ERK. Results are representative of three different experiments. Error bars indicate ±SD. (C) NK cell cytotoxicity experiments were performed after transfection of ARK cells with ERK. Results are representative of three different experiments. Error bars indicate ±SD. (D) Ab blocking experiments were performed to demonstrate the role of NKG2D/NKG2D ligand interaction in NK cell lysis after transfection of ARK cells with ERK. Results are representative of three different experiments. Error bars indicate ±SD. *$P < .05$; **$P > .05$.

associated with tumor eradication and increased patient survival in both experimental animals and human cancer patients [9,23,24]. NKG2D recognizes ligands that are often expressed at higher levels on tumor cells than in the surrounding normal tissue and that can be induced further by cancer treatments. Increasing evidence has shown that some chemotherapeutic agents, such as HSP-90 inhibitors, genotoxic agents, HDAC inhibitors, or proteasome inhibitors (e.g., bortezomib) can increase the expression of NKG2D ligands, thus facilitating the activation of NKG2D-expressing lymphocytes [including NK cells, NKT cells, and cytotoxic T lymphocytes (CTLs)] and tumor cell lysis. Moreover, activation of the DNA damage response by chemotherapy or radiotherapy can also increase the expression of death receptors in several tumors, facilitating the cytotoxic action of Fas-L– and TRAIL-positive immune effectors on tumor cells [25–27]. Therefore, effective cancer treatments might both directly damage tumor cells and induce NKG2D ligand expression and subsequent attacks by killer lymphocytes. The expression of NKG2D ligand has been described in multiple types of tumor including MM [28,29]. We investigated NKG2D ligand regulation in human myeloma after HDAC inhibitor VPA treatment.

In this study, we found that VPA treatment induced MICA/B and ULBP2 mRNA and protein expressions in ARK, OPM2, and OCI-MY5 myeloma cell lines. We also found that VPA has the same effects on MICA/B and ULBP2 mRNA and protein expressions in patients' primary myeloma cells. However, surface expression levels of other NK cell activating receptor ligands including NKp30-L, NKp44-L, NKp46-L, CD48, CS1, CD155, CD112, CD28, AICL(CLEC2B), and LFA-3(CD58) were not significantly influenced by VPA treatment in ARK cells. Previously, a number of studies have shown that

HDAC inhibitors were able to increase expression of NKG2D ligands in a variety of hematological and non-hematological malignant models [8,10,15,30,31]. Kato et al. [10] found that treatment with HDAC inhibitor trichostatin A induced MICA and MICB mRNA and protein expressions in both lymphoid leukemic cells and myeloid leukemic cells. Armeanu et al. [8] reported that the HDAC inhibitor VPA induced the expression of MICA and MICB in hepatocellular carcinoma. These results indicate that the expression of NKG2D ligands was at least partly regulated by histone acetylation.

NKG2D ligands are induced by a wide variety of signals that have collectively been termed "cell stress" [32–35]. DNA damage response pathways mediated by mutated ataxia-telangiectasia (ATM) or ATM- and Rad3-related (ATR) protein kinases have been reported to upregulate NKG2D ligand expression [35–37]. Gasser et al. demonstrated that NKG2D ligand expression was induced by ATM/ATR signaling in the DNA damage response pathway, which was prevented by ATM/ATR inhibitors, such as caffeine [35]. Sp1 family members (also referred to as the SP1-related retinoblastoma control proteins) have been implicated in ULBP1, MICA, and MICB transcriptions [38–40]. López-Soto et al. proposed that the transcription is controlled by competition between stimulatory Sp3 and repressive AP-2α transcription factors at a cyclic adenosine monophosphate response element–like site in the ULBP1 promoter [38]. Butler et al. demonstrated that p53 family members are important in proteasome inhibitor drug-induced ULBP1 up-regulation in head and neck squamous cell carcinoma [41]. The activation of the heat shock factor-1 has also been shown to regulate MICA/B expression [39]. However, it was reported that cell regulatory pathways such as ERK or AKT may influence NKG2D ligand expression of leukemic cells [42–45].

To investigate the signaling pathways involved in the regulation of NKG2D ligand expression on myeloma cells after exposure to VPA, we focused our attention on ERK and AKT pathways. In this study, we show for the first time, by Western blot, that VPA treatment of myeloma cells leads to a stronger constitutive pERK than that of the control cells. Interestingly, we observed the increased expression of pERK1 and the loss of pERK2 upon treatment with VPA. The underlying mechanism might be due to loss of competition between ERK1 and ERK2 for their binding and activation by mitogen-activated protein kinases. Consistent with our observations, it has been previously reported that the ERK2 knockdown was accompanied by an increase in ERK1 phosphorylation in the mouse spinal cord dorsal horn [46]. Frémin et al. [47] also observed an increase in ERK1 phosphorylation level in ERK2-silenced rat and mouse hepatocytes. Mazzucchelli et al. [48] showed that ERK1 knockout could be accompanied by an increase in ERK2 signaling. However, the reason for the preferential phosphorylation of ERK1 in VPA-treated myeloma cells needs to be further investigated. Our results also demonstrate that PD98059, the inhibitor of ERKs, blocks induction of MICA/B and ULBP2 expressions on VPA-treated ARK cells and impaired NK cell lysis. Furthermore, transfecting ARK cells with constitutively active ERK induced significantly higher MICA/B and ULBP2 expressions, when compared with parental ARK cells. It also increased NK cell lysis. These findings suggest that ERK activation is involved in the up-regulation of NKG2D ligand, leading to increased sensitivity of VPA-treated myeloma cells to NK cell lysis. Inconsistent with our data, ERK was also shown to increase the expression of NKG2D ligands and the sensitivity to NK cell–mediated lysis of transformed cells derived from solid tumors and leukemia after H_2O_2-induced oxidative stress [20].

The interaction between NKG2D and its ligands potentially plays a central role in antitumor surveillance. The levels of NKG2D ligands might determine the strength of antitumor immune responses [32,49]. Some factors, such as heat shock, transformation, and viral or bacterial infection, have been found to regulate NKG2D ligand expression. It has been recently shown that treatment with HDAC inhibitor induces MICA or MICB expression on some tumor cells, rendering them more sensitive to NK cell–mediated killing [8,10]. In our study, we also demonstrated that the expression of activating ligands for NKG2D in myeloma cells could be induced by HDAC inhibitor VPA, leading to the enhancement of NK cell degranulation and an increased susceptibility of myeloma cell lines and patients' primary myeloma cells to the cytotoxicity of NK cells. Perforin/granzyme- and Fas-based cytolytic pathways are two major mechanisms of cell-mediated cytotoxicity. We found that the primary mechanism of NK cell cytolysis was perforin/granzyme-mediated and not through the death receptor pathway. Moreover, this enhanced cytotoxicity was critically dependent on the interaction between NKG2D and its ligands because it was abolished by addition of anti-NKG2D mAb. Taken together, these observations support the hypothesis that the induction of NKG2D ligand expression renders myeloma cells susceptible to NK cell–mediated killing, which can be markedly enhanced by VPA treatment.

In the present study, we demonstrated that ERK signaling pathway was involved in VPA-induced expression of NKG2D ligands of human myeloma cells. We also provided evidence that VPA treatment could induce expression of NKG2D ligands in myeloma cells and consequently enhanced the sensitivity of myeloma cells to NK cell–mediated lysis through a perforin/granzyme-mediated mechanism. Furthermore, the increase of NK cell lytic activity was significantly dependent on NKG2D activation. Our data suggested that the enhancement of NKG2D ligand expressions using HDAC inhibitor may be an attractive therapeutic strategy to induce the susceptibility of myeloma cells to NK cell–mediated killing. The ERK pathway leading to up-regulation of NKG2D ligands may be a productive target for design of therapeutic agents to enhance the immunogenicity of tumor cells.

References

[1] Mahindra A, Laubach J, Raje N, Munshi N, Richardson PG, and Anderson K (2012). Latest advances and current challenges in the treatment of multiple myeloma. *Nat Rev Clin Oncol* **9**, 135–143.

[2] Mohty B, El-Cheikh J, Yakoub-Agha I, Avet-Loiseau H, Moreau P, and Mohty M (2012). Treatment strategies in relapsed and refractory multiple myeloma: a focus on drug sequencing and 'retreatment' approaches in the era of novel agents. *Leukemia* **26**, 73–85.

[3] Topalian SL, Weiner GJ, and Pardoll DM (2011). Cancer immunotherapy comes of age. *J Clin Oncol* **29**, 4828–4836.

[4] Ruggeri L, Zhang S, and Farag SS (2009). Natural killer cell activity and killer immunoglobulin-like receptors in hematopoietic stem cell transplantation. *Cancer Treat Res* **144**, 47–69.

[5] Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, and Spies T (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285**, 727–729.

[6] Cosman D, Müllberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, and Chalupny NJ (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14**, 123–133.

[7] Watson NF, Spendlove I, Madjd Z, McGilvray R, Green AR, Ellis IO, Scholefield JH, and Durrant LG (2006). Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients. *Int J Cancer* **118**, 1445–1452.

[8] Armeanu S, Bitzer M, Lauer UM, Venturelli S, Pathil A, Krusch M, Kaiser S, Jobst J, Smirnow I, Wagner A, et al. (2005). Natural killer cell–mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer Res* **65**, 6321–6329.

[9] Conejo-Garcia JR, Benencia F, Courreges MC, Gimotty PA, Khang E, Buckanovich RJ, Frauwirth KA, Zhang L, Katsaros D, Thompson CB, et al. (2004). Ovarian carcinoma expresses the NKG2D ligand Letal and promotes the survival and expansion of $CD28^-$ antitumor T cells. *Cancer Res* **64**, 2175–2182.

[10] Kato N, Tanaka J, Sugita J, Toubai T, Miura Y, Ibata M, Syono Y, Ota S, Kondo T, Asaka M, et al. (2007). Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of leukemic cells to the cytotoxicity of NKG2D-expressing cells. *Leukemia* **21**, 2103–2108.

[11] Skov S, Pedersen MT, Andresen L, Straten PT, Woetmann A, and Odum N (2005). Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3–dependent expression of MHC class I–related chain A and B. *Cancer Res* **65**, 11136–11145.

[12] Tieng V, Le Bouguénec C, du Merle L, Bertheau P, Desreumaux P, Janin A, Charron D, and Toubert A (2002). Binding of *Escherichia coli* adhesin AfaE to CD55 triggers cell-surface expression of the MHC class I-related molecule MICA. *Proc Natl Acad Sci USA* **99**, 2977–2982.

[13] Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, and Spies T (2001). Costimulation of CD8αβ T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol* **2**, 255–260.

[14] Blaheta RA and Cinatl J Jr (2002). Anti-tumor mechanisms of valproate: a novel role for an old drug. *Med Res Rev* **22**, 492–511.

[15] Diermayr S, Himmelreich H, Durovic B, Mathys-Schneeberger A, Siegler U, Langenkamp U, Hofsteenge J, Gratwohl A, Tichelli A, Paluszewska M, et al. (2008). NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. *Blood* **111**, 1428–1436.

[16] Lu X, Ohata K, Kondo Y, Espinoza JL, Qi Z, and Nakao S (2010). Hydroxyurea upregulates NKG2D ligand expression in myeloid leukemia cells synergistically with valproic acid and potentially enhances susceptibility of leukemic cells to natural killer cell-mediated cytolysis. *Cancer Sci* **101**, 609–615.

[17] Cinatl J Jr, Cinatl J, Scholz M, Driever PH, Henrich D, Kabickova H, Vogel JU, Doerr HW, and Kornhuber B (1996). Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. *Anticancer Drugs* **7**, 766–773.

[18] Mandelboim O, Malik P, Davis DM, Jo CH, Boyson JE, and Strominger JL (1999). Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity. *Proc Natl Acad Sci USA* **96**, 5640–5644.

[19] Bryceson YT, March ME, Barber DF, Ljunggren HG, and Long EO (2005). Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med* **202**, 1001–1012.

[20] Borchers MT, Harris NL, Wesselkamper SC, Vitucci M, and Cosman D (2006). NKG2D ligands are expressed on stressed human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **291**, L222–L231.

[21] Boissel N, Rea D, Tieng V, Dulphy N, Brun M, Cayuela JM, Rousselot P, Tamouza R, Le Bouteiller P, Mahon FX, et al. (2006). BCR/ABL oncogene directly controls MHC class I chain-related molecule A expression in chronic myelogenous leukemia. *J Immunol* **176**, 5108–5116.

[22] Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM, and Hayakawa Y (2005). NKG2D function protects the host from tumor initiation. *J Exp Med* **202**, 583–588.

[23] Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, Knoblaugh S, Cado D, Greenberg NM, and Raulet DH (2008). NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* **28**, 571–580.

[24] Friese MA, Wischhusen J, Wick W, Weiler M, Eisele G, Steinle A, and Weller M (2004). RNA interference targeting transforming growth factor-β enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity *in vivo*. *Cancer Res* **64**, 7596–7603.

[25] Zitvogel L, Apetoh L, Ghiringhelli F, and Kroemer G (2008). Immunological aspects of cancer chemotherapy. *Nat Rev Immunol* **8**, 59–73.

[26] Ullrich E, Bonmort M, Mignot G, Kroemer G, and Zitvogel L (2008). Tumor stress, cell death and the ensuing immune response. *Cell Death Differ* **15**, 21–28.

[27] Zitvogel L, Apetoh L, Ghiringhelli F, André F, Tesniere A, and Kroemer G (2008). The anticancer immune response: indispensable for therapeutic success? *J Clin Invest* **118**, 1991–2001.

[28] González S, Groh V, and Spies T (2006). Immunobiology of human NKG2D and its ligands. *Curr Top Microbiol Immunol* **298**, 121–138.

[29] Okamoto M, Inaba T, Yamada N, Uchida R, Fuchida SI, Okano A, Shimazaki C, and Taniwaki M (2006). Expression and role of MHC class I-related chain in myeloma cells. *Cytotherapy* **8**, 509–516.

[30] Poggi A, Catellani S, Garuti A, Pierri I, Gobbi M, and Zocchi MR (2009). Effective *in vivo* induction of NKG2D ligands in acute myeloid leukaemias by all-trans-retinoic acid or sodium valproate. *Leukemia* **23**, 641–648.

[31] Chávez-Blanco A, De la Cruz-Hernández E, Domínguez GI, Rodríguez-Cortez O, Alatorre B, Pérez-Cárdenas E, Chacón-Salinas R, Trejo-Becerril C, Taja-Chayeb L, Trujillo JE, et al. (2011). Upregulation of NKG2D ligands and enhanced natural killer cell cytotoxicity by hydralazine and valproate. *Int J Oncol* **39**, 1491–1499.

[32] Raulet DH (2003). Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* **3**, 781–790.

[33] Gleimer M and Parham P (2003). Stress management: MHC class I and class I-like molecules as reporters of cellular stress. *Immunity* **19**, 469–477.

[34] Unni AM, Bondar T, and Medzhitov R (2008). Intrinsic sensor of oncogenic transformation induces a signal for innate immunosurveillance. *Proc Natl Acad Sci USA* **105**, 1686–1691.

[35] Gasser S, Orsulic S, Brown EJ, and Raulet DH (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* **436**, 1186–1190.

[36] Cerboni C, Zingoni A, Cippitelli M, Piccoli M, Frati L, and Santoni A (2007). Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK-cell lysis. *Blood* **110**, 606–615.

[37] Molinero LL, Fuertes MB, Girart MV, Fainboim L, Rabinovich GA, Costas MA, and Zwirner NW (2004). NF-κB regulates expression of the MHC class I-related chain A gene in activated T lymphocytes. *J Immunol* **173**, 5583–5590.

[38] López-Soto A, Quiñones-Lombraña A, López-Arbesú R, López-Larrea C, and González S (2006). Transcriptional regulation of ULBP1, a human ligand of the NKG2D receptor. *J Biol Chem* **281**, 30419–30430.

[39] Venkataraman GM, Suciu D, Groh V, Boss JM, and Spies T (2007). Promoter region architecture and transcriptional regulation of the genes for the MHC class I-related chain A and B ligands of NKG2D. *J Immunol* **178**, 961–969.

[40] Zhang C, Wang Y, Zhou Z, Zhang J, and Tian Z (2009). Sodium butyrate upregulates expression of NKG2D ligand MICA/B in HeLa and HepG2 cell lines and increases their susceptibility to NK lysis. *Cancer Immunol Immunother* **58**, 1275–1285.

[41] Butler JE, Moore MB, Presnell SR, Chan HW, Chalupny NJ, and Lutz CT (2009). Proteasome regulation of ULBP1 transcription. *J Immunol* **182**, 6600–6609.

[42] Bortul R, Tazzari PL, Billi AM, Tabellini G, Mantovani I, Cappellini A, Grafone T, Martinelli G, Conte R, and Martelli AM (2005). Deguelin, A PI3K/AKT inhibitor, enhances chemosensitivity of leukaemia cells with an active PI3K/AKT pathway. *Br J Haematol* **129**, 677–686.

[43] Grandage VL, Gale RE, Linch DC, and Khwaja A (2005). PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-κB, Mapkinase and p53 pathways. *Leukemia* **19**, 586–594.

[44] Yin B, Morgan K, Hasz DE, Mao Z, and Largaespada DA (2006). *Nfl* gene inactivation in acute myeloid leukemia cells confers cytarabine resistance through MAPK and mTOR pathways. *Leukemia* **20**, 151–154.

[45] Ogbomo H, Michaelis M, Klassert D, Doerr HW, and Cinatl J Jr (2008). Resistance to cytarabine induces the up-regulation of NKG2D ligands and enhances natural killer cell lysis of leukemic cells. *Neoplasia* **10**, 1402–1410.

[46] Xu Q, Garraway SM, Weyerbacher AR, Shin SJ, and Inturrisi CE (2008). Activation of the neuronal extracellular signal-regulated kinase 2 in the spinal cord dorsal horn is required for complete Freund's adjuvant-induced pain hypersensitivity. *J Neurosci* **28**, 14087–14096.

[47] Frémin C, Ezan F, Boisselier P, Bessard A, Pagès G, Pouysségur J, and Baffet G (2007). ERK2 but not ERK1 plays a key role in hepatocyte replication: an RNAi-mediated ERK2 knockdown approach in wild-type and ERK1 null hepatocytes. *Hepatology* **45**, 1035–1045.

[48] Mazzucchelli C, Vantaggiato C, Ciamei A, Fasano S, Pakhotin P, Krezel W, Welzl H, Wolfer DP, Pagès G, Valverde O, et al. (2002). Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. *Neuron* **34**, 807–820.

[49] López-Larrea C, Suárez-Alvarez B, López-Soto A, López-Vázquez A, and Gonzalez S (2008). The NKG2D receptor: sensing stressed cells. *Trends Mol Med* **14**, 179–189.